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## PROTEIN L AND HYBRID PROTEINS THEREOF

### BACKGROUND OF THE INVENTION

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#### Field of the Invention

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The present invention relates to sequences of protein L which bind to light  
5 chains of immunoglobulins. The invention also relates to hybrid proteins of protein L  
having the ability to bind to light chains of all Ig and also to bind to light and heavy chains  
of immunoglobulin G, DNA-sequences which code for the proteins vectors that contain  
such DNA-sequences, host cells transformed by the vectors, methods for preparing the  
proteins, reagent apparatus for separating and identifying immunoglobulins, compositions  
10 and pharmaceutical compositions which contain the proteins.

#### Description of the Related Art

The invention relates in particular to the DNA-sequence and to the amino  
acid sequence of the light-chain forming domains of protein L.

Proteins which bind to the constant domains (of high affinity) of the  
15 immunoglobulins (Ig) are known. Thus, protein A (from *Staphylococcus aureus*)  
(Forsgren, A. and Sjöquist, J. (1966) Protein A from *Staphylococcus aureus*. I. Pseudo-  
immune reaction with human gamma-globulin. J. Immunol. 97: 822-827) binds to IgG  
from various mammal species. The binding of protein A to IgG is mediated essentially via  
surfaces in the Fc-fragment of the heavy chain of the IgG-molecule, although a certain  
20 bond is also effected with surfaces in the Fab-fragment of the IgG. Protein A lacks the  
ability of binding to human IgG3 and neither will it bind to IgG from several other animal  
species, such as important laboratory animals, for instance rats and goats, which limits the  
use of protein A.

Protein G (Björck, L. and Kronvall, G. (1984) Purification and some  
25 properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. 133: 969-

974; Reis, K., Ayoub, E. and Boyle, M. (1984) Streptococcal Fc receptors. I. Isolation and partial characterization of the receptor from a group C streptococcus. J. Immunol. 132: 3091-3097) binds to heavy chains in human IgG and to all four of its subclasses and also to IgG from most mammals, including rats and goats.

5 Protein H (Åkesson, P., Cooney, J., Kishimoto, F. and Björck, L. (1990) Protein H - a novel IgG binding bacterial protein. Molec. Immun. 27: 523-531) binds to the Fc-fragment in IgG from human beings, monkeys and rabbits. However, the bond is weaker than in the case of protein G and A, which may be beneficial when wishing to break the bond with a weak agent, for instance when purifying proteins which are readily  
10 denatured with the aid of antibodies.

Protein M (Applicant's Patent Application PCT/SE 91100447) binds to the Fc-fragment in IgG from humans, monkeys, rabbits, goats, mice and pigs.

Protein L (Björck, L. (1988) Protein L, a novel bacterial cell wall protein with affinity to Ig L chains. J. Immunol. 140: 1194-1197), which binds to the light chains  
15 in immunoglobulins from all of the classes G, A, M, D and E is known (USP 4,876,194). The amino acid sequence and the binding domains of this protein, however, have hitherto been unknown.

The aforesaid proteins can be used in the analysis, purification and preparation of antibodies and for diagnostic and biological research.

20 The elimination of immunoglobulins, with the aid of plasmapheresis, can have a favourable effect on some autoimmune diseases. A broadly binding protein would be an advantage when wishing to eliminate all classes of antibodies in this context.

It has long been known that infectious conditions can be prevented or cured with the introduction of an immune serum, i.e. a serum which is rich in antibodies against  
25 the organism concerned or its potentially harmful product. Examples hereof are epidemic jaundice, tetanus, diphtheria, rabies and generalized shingles. Antibodies against a toxic product may also be effective in the case of non-infectious occasioned conditions. Serum produced in animals against different snake venoms is the most common application in this respect. However, the administration of sera or antibody preparations is not totally without

risk. Serious immunological reactions can occur in some cases. Singular cases of the transmission of contagious diseases, such as HIV and hepatitis through the agency of these products have also been described. In order to avoid these secondary effects, it has been desirable to produce therapeutic antibodies in test tubes. A large number of novel techniques for the preparation of antibodies in test tubes have been proposed in recent years. Examples of such techniques are hybridom techniques, synthesis of chima-antibodies and the preparation of antibodies in bacteria. These techniques also enable antibodies to be specially designed which can further widen the use of such molecules as therapeutics, for instance in the case of certain tumour-diseases. In the case of some of these novel methods, however, the product totally lacks the Fc-fragment to which all of the described IgG-binding proteins, with the exception of protein L, bind. There is consequently a need of a process for purifying antibodies for therapeutic use, wherein proteins which have a broad binding activity/specificity, can be of value.

It has long been possible to utilize the antibody reaction with its high grade specificity for diagnosing past or, in some cases, ongoing infections with different parasites. This indirect method of indicating infectious agents is called serology and, in many cases, may be the only diagnostic alternative. In certain cases, it can also be of interest to exhibit specific IgE- or IgA-antibodies. When diagnosing with the aid of serology, the antigen is most often fastened to a solid phase, whereafter serum taken from the patient is incubated with the antigen. Antibodies that have been bound from the patient can then be detected in different ways, often with the aid of a secondary antibody (for instance, an antibody which is directed against the light chains of human antibodies) to which an identifiable label has been attached, such as alkaline phosphatase, biotin, radioactive isotopes, fluorescein, etc. In this context, a protein having a broad Ig binding capacity can be used as an alternative to secondary antibodies.

There are a number of non-therapeutic and non-diagnostic reasons for the necessity to bind antibodies. Antibodies are often used in research, both for detection and for purifying the antigen against which they are directed. All techniques which facilitate

the purification of antibodies and, in particular, techniques which enable different classes to be purified, are of interest in this context.

Consequently, there is a serious need of a protein which has a broad binding activity/specificity and which binds to several different classes of immunoglobulins from  
5 different animal species. At present, there is no known protein which will bind to all immunoglobulin classes. The earlier known proteins A, G, H and M bind only to heavy chains in IgG.

#### BRIEF SUMMARY OF THE INVENTION

The known protein L (Björck et al, 1988) binds to the light  $\chi$ -chains and  $\gamma$ -  
10 chains in immunoglobulins of all classes, although the bonds are much weaker on the  $\kappa$ -chains. Applicant has charted protein L, has determined the amino acid sequence for protein L, has identified the light-chain binding domains on protein L, and has used these to produce hybrid proteins which possess the IgG-Fc-binding domains of protein G. The Applicant is able to show through protein LG that a protein of broader binding activity/  
15 specificity can be produced thereby. The aforesaid proteins A, G, H and M bind to the same surfaces, or to very closely lying surfaces on IgG-Fc. The protein L which binds to light chains can thus be combined with any other functionally similar protein which binds to the Fc-fragment of heavy chains. A similar broadening of the Ig-binding activity is achieved with all alternatives.

#### 20 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 illustrates the ~~plasmid~~ ~~pHD389~~; the ribosomal binding sequence, the sequence for the signal peptide from ~~ompA~~ and recognition sequence for several restriction enzymes are shown;

~~Figure 2 illustrates the amino acid and nucleic acid sequence for protein LG.~~

Figure 3 is a schematic overall view of the production of protein L.

Figure 4 is a schematic overall view of the production of protein LG.

Figures 5a, 5b and 5c are schematic overall views of the production of the hybrid proteins LA, LM and LH respectively.

Figure 6 is a schematic inclusive illustration of protein A, G, H and M1. IgGFc-binding domains are for protein A: E, D, A, B and C; for protein G: C1, C2 and C3; for protein H: A and/or B; and for protein M1: A, B1, B2, B3 and S.

~~Figure 7 illustrates the amino acid and nucleic acid sequence for protein M1.~~

Figure 8 illustrates Western Blot for protein G, L and LG with certain immunoglobulins and immunoglobulin fragments.

Figure 9 illustrates Slot-Blot for protein L, G and LG with IgG, Ig $\chi$  and Ig Fc.

The amino acid and nucleic acid sequence of the light-chain binding domains of protein L is illustrated in Figure 2.

It will be observed that the drawings are not to scale.

#### DETAILED DESCRIPTION OF THE INVENTION

Thus, the present invention relates to the sequence of protein L which binds to light chains in Ig and has the amino acid sequence disclosed in Figure 1, and variants, subfragments, multiples or mixtures of the domains B1-B5 having the same binding properties. The invention also relates to a DNA-sequence which codes for such protein sequences, for instance the DNA-sequence in Figure 1.

The invention is concerned with a hybrid protein which is characterized by comprising domains which bind to the light  $\chi$ -chains and  $\lambda$ -chains in immunoglobulins of all classes, and also comprises domains which bind to heavy chains in immunoglobulin G, wherein those domains which bind to the light chains are chosen from among the B1-, B2-, B3-, B4- and B5-domains in protein L and those domains which bind to heavy chains of immunoglobulins are chosen from the C1-, C2- and C3-domains in protein G; the A-, B- and C1-domains from protein H; the A-, B1-, B2- and S-domains in protein M1 or the E-, D-, A-, B- and C-domains in protein A (see Figure 6) and variants, subfragments, multiples

or mixtures of these domains that have the same binding properties which bind to heavy chains of immunoglobulins.

By subfragment is meant a part-fragment of the given domains or fragments which include parts from the various domains having mutually the same binding properties.

5 By variants is meant proteins or peptides in which the original amino acid sequence has been modified or changed by insertion, addition, substitution, inversion or exclusion of one or more amino acids, although while retaining or improving the binding properties. The invention also relates to those proteins which contain several arrays (multiples) of the binding domains or mixtures of the binding domains with retained binding properties. The  
10 invention also relates to mixtures of the various domains of amino acid sequences having mutually the same binding properties.

The invention relates in particular to a hybrid protein designated LG, and is characterized in that the hybrid protein includes the B-domains in protein L which bind to the light chains in immunoglobulins, and the C1- domains and C2-domains in protein G  
15 which bind to heavy chains and have the amino acid sequence disclosed in Figure 2. The invention also relates to variants, subfragments, multiples or mixtures of these domains.

Protein LG is a hybrid protein having a molecular weight of about 50 kDa (432 amino acids) and comprising four domains, each of which binds to light chains in immunoglobulins, and two IgG-binding domains from protein G. The hybrid protein  
20 combines a broad IgG-binding activity, deriving from the high-grade binding ability of protein G to the Fc-fragment of the heavy chain on IgG with the ability of the protein L to bind to light chains of all classes of immunoglobulins. Thus, protein LG binds polyclonal human IgG, IgM, IgA, IgD and IgE. The affinity for human polyclonal IgG is  $2 \times 10^{10} \text{ M}^{-1}$ . All four human immunoglobulin classes are bound. Binding to human IgG is effected with  
25 both the  $\kappa$ -and the  $\lambda$ -chain. Both the Fc-fragment and the Fab-fragment of IgG are bound to the hybrid protein. The protein also binds human IgA-, IgD-, IgE- and IgM-antibodies. The bond is stronger to human immunoglobulins which carry  $\chi$  than to those which carry the  $\lambda$ -isotope of light chains. IgG from most mammals will be bound by protein LG, thus also IgG from goats and cows, which do not bind to protein L. However, rabbit-IgG which

binds relatively weakly to protein L will bind well to the fusion protein. IgM and IgA-antibodies from mice, rats and rabbits will be bound to the protein. Protein LG is highly soluble. It is able to withstand heat and will retain its binding properties even at high temperatures. The binding properties also remain in a broad pH-range of 3-10. The protein withstands detergent and binds marked or labelled proteins subsequent to separation in SDS-PAGE and transference to membranes with elektroblotting. The protein can be immobilized on a solid phase (nitrocellulose, Immobilon<sup>®</sup>, polyacrylamide, plastic, metal and paper) without losing its binding capacity. The binding properties are not influenced by marking with radioactive substances, biotin or alkaline phosphatase. (The binding abilities of the protein LG are disclosed in Example 3).

The protein comprises 432 amino acids and has a molecular weight of 50 kDa deriving therefrom. The sequence is constructed of an ala sequence of the three last amino acids in the A-domain of the protein L (val-glu-asn), this ala sequence being unrelated to the two proteins, whereafter the four mutually high-grade homologous B-domains from protein L follow. The first of the B-domains is comprised of 76 amino acids, and the remaining domains are each comprised of 72 amino acids. The first nine amino acids from the fifth B-domain are included and followed by two non-related amino acids (pro-met). The protein G-sequences then follow. The last amino acid in the so-called S-domain from protein G is followed by an IgG binding domain from protein G (C1; 55 amino acids), the intermediate D-region (15 amino acids) and the second IgG-binding C-domain (C2; 55 amino acids). The last amino acid is a methionine, which occurs in natural protein G as the first amino acid in the so-called W-region.

The invention also relates to DNA-sequences which code for the aforesaid proteins.

The gene which codes for the IgG-binding amino acid sequences can be isolated from the chromosomal DNA from *Staphylococcus aureus* based on the information on the DNA-sequence for protein A (S. Löfdahl, B. Guss, M. Uhlen, L. Philipsson and M. Lindberg. 1983. Gene for staphylococcal protein A. Proc. Natl. Acad. Sci. USA. 80: 697-701) and Figure 6, or from G-streptococcus, preferably strain G 148 or

C-streptococcus, preferably strain *Streptococcus equisimilis* C 40, based on the information on protein G (B. Guss, M. Eliasson, A. Olsson, M. Uhlen, A.-K. Frej, H. Jörvall, I. Flock and M. Lindberg. 1986. Structure of the IgG-binding regions of streptococcal protein G. EMBO. J. 5: 1567-1575) and Figure 6, or from group A-streptococcus, e.g. *S. pyogenes* (type M1) based on the information on the DNA-sequence for protein H (H. Gomi, T. Hozumi, S. Hattori, C. Tagawa, F. Kishimoto and L. Björck. 1990. The gene sequence and some properties of protein H - a novel IgG binding protein J. Immunol. 144: 4046-4052) and Figure 6, or from the chromosomal DNA in group A-streptococcus type M1 based on the information on the DNA-sequence for protein M (Applicant's Patent Application, PCT/SE 91100447) and Figures 6 and 7. The gene which codes for the protein that binds to light chains can be isolated from the chromosomal DNA from *Peptostreptococcus magnus* 312 based on the information on the DNA-sequence for protein L in Figure 2.

By using the chromosomal DNA obtained from the aforesaid bacteria as a template, a DNA-fragment defined with the aid of two synthetic oligonucleotides can then be specifically amplified with the aid of PCR (Polymerase Chain Reaction). This method also enables recognition sites to be incorporated for restriction enzymes in the ends of the amplified fragments (PCR technology, Ed: PCR Technology. Principles and Applications for DNA Amplification. Ed. Henry Erlich. Stockton Press, New York, 1989). The choice of recognition sequences can be adapted in accordance with the vector chosen to express the fragment or the DNA-fragment or other DNA-fragments with which the amplified fragment is intended to be combined. The amplified fragment is then cleaved with the restriction enzyme or enzymes concerned and is combined with the fragment/the other fragments concerned and the fragments are then cloned together in the chosen vector (in this case, the expression vector) (Sambrook, J.E. Fritsch and T. Maniatis, 1989, Molecular cloning: A laboratory manual, 2nd Ed. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, USA). The plasmid vector pHD313 can be used (Dalbøge, H.E. Bech Jensen, H. Töttrup, A. Grubb, K. Abrahamson, I. Olafsson and S. Carlsen, 1989. High-level expression of active human cystatin C in *Escherichia coli*. Gene, 79: 325-332),



alternatively one of the vectors in the so-called PET-series (PET 20, 21, 22, 23) retailed by Novagen (Madison, Wisconsin, USA).

The hybrid proteins are then incorporated in an appropriate host, preferably *E. coli*. The invention also relates to such hosts as those in which the hybrid proteins are  
5 incorporated.

Those clones which produce the desired proteins can be selected from the resultant transformants with the aid of a known method (Fahnestock et al., *J. Bacteriol.* 167, 870 (1986).

When the proteins that can bind to the light chains in the immunoglobulins  
10 and to the heavy chains in IgG have been purified from the resultant positive clones with the aid of conventional methods, the binding specificities of the proteins are determined for selection of those clones which produce a protein that will bind to the light chains in immunoglobulins and to the heavy chains in IgG.

Subsequent to having isolated plasmid DNA in said clone with conventional  
15 methods, the DNA-sequence in the inserted material is determined with known methods (Sanger et al., *Proc. Natl. Acad. Sci. USA* 74, 5463 (1977).

The invention also relates to DNA-sequences which hybridize with said identified DNA-sequences under conventional conditions and which code for a protein that possesses the desired binding properties. Strict hybridizing conditions are preferred.

20 Expression of the genes can be effected with expression vectors which have the requisite expression control regions, the structural gene being introduced after said regions. As illustrated in Figure 1 and Claim 2, the structural gene can be used for protein LG or other hybrid proteins with protein L.

With regard to expression vectors, different host-vector-systems have been  
25 developed, of which the most suitable host-vector-systems can be selected for expression of the genes according to the present invention.

The present invention also relates to a method of producing the inventive hybrid proteins by cultivating a host cell which is transformed with an expression vector in which DNA which codes for the proteins according to the invention is inserted.

This method includes the steps of

(1) inserting into a vector a DNA-fragment which codes for the hybrid proteins;

(2) transforming the resultant vector into an appropriate host cell;

5 (3) cultivating the resultant, transformed cell for preparation of the desired hybrid protein; and

(4) extracting the protein from the culture.

In the first step, the DNA-fragment which codes for the hybrid protein is inserted in a vector which is suitable for the host that is to be used to express the hybrid protein. The gene can be inserted by cleaving the vector with an appropriate restriction  
10 enzyme, and then ligating the gene with the vector.

In the second step, the vector with the hybrid plasmid is inserted into host cells. The host cells may be *Escherichia coli*, *Bacillus subtilis* or *Saccharomyces cerevisiae* or other suitable cells. Transformation of the expression's hybrid vector into the  
15 host cell can be effected in a conventional manner and clones which have been transformed can then be selected.

In the third step, the obtained transformants are cultivated in an appropriate medium for preparation of the desired proteins by expression of the gene coded for the hybrid protein.

20 In the fourth step, the desired protein is extracted from the culture and then purified. This can be achieved with the aid of known methods. For instance, the cells can be lysed with the aid of known methods, by treating the cells with ultrasonic sound, enzymes or by mechanical degradation. The protein which is released from the cells or which excretes in the medium can be recovered and purified with the aid of conventional  
25 methods often applied within the biochemical field, such as ion-exchange chromatography, gel filtration, affinity chromatography with the use of immunoglobulins as ligands, hydrophobic chromatography or reverse-phase chromatography. These methods can be applied individually or in suitable combinations.

As mentioned previously, the inventive proteins may be used for binding, identifying or purifying immunoglobulins. They can also be bound to pharmaceuticals and used in formulations which have delayed release properties. To this end, the protein may be present in a reagent appliance for pharmaceutical composition in combination with appropriate reagents, additives or carriers.

The proteins can be handled in a freeze-dried state or in a PBS-solution (phosphate-buffered physiological salt solution) pH 7.2 with 0.02%  $\text{NaN}_3$ . It can also be used connected to a solid phase, such as carbohydrate-based phases, for instance CNBr-activated sepharose, agarose, plastic surfaces, polyacrylamide, nylon, paper, magnetic spheres, filter, films. The proteins may be marked with biotin, alkaline phosphatase, radioactive isotopes, fluorescein and other fluorescent substances, gold particles, ferritin, and substances which enable luminescence to be measured.

Other proteins may also be used as carriers. These carriers may be bound to or incorporated in the proteins, in accordance with the invention. For instance, it is conceivable to consider the whole of proteins A, G, H, M as carriers for inserted sequences of protein L which bind to light chains. In turn, these carriers can be bound to the aforesaid carriers.

The pharmaceutical additions that can be used are those which are normally used within this field, such as pharmaceutical qualities of mannitol, lactose, starch, magnesium stearate, sodium saccharate, talcum, cellulose, glyucose, gelatine, saccharose, magnesium carbonate and similar extenders, such as lactose, dicalcium phosphate and the like; bursting substances, such as starch or derivatives thereof; lubricants such as magnesium stearate and the like; binders, such as starch, gum arabicum, polyvinylpyrrolidone, gelatine, cellulose and derivatives thereof, and the like.

The invention will now be described in more detail with reference to the accompany drawings.

Example 1Cloning and expression of the IgG-light-chain-binding domains in Protein L**Construction of synthetic oligonucleotides (primers) for amplifying sequences coded for protein L, domain B1-B4**

5 ~~It has been found that a protein L-peptide (expressed in *E. coli*) constructed~~  
 of the sequence ala-val-glu-asn domain B1 (from protein L) binds to the light chains of the  
 immunoglobulins (W. Kastern, U. Sjöbring and L. Björck. 1992. Structure of  
 peptostreptococcal protein L and identification of a repeated immunoglobulin light chain-  
 binding domain. J. Biol. Chem. 267 (18):12820-5). Since this simple protein L-domain  
 10 has a relatively low affinity to Ig, ( $1 \times 10^7 \text{ M}^{-1}$ ), and since the naturally occurring protein L  
 which is constructed of several mutually similar domains (B1-B5) has a high affinity to Ig  
 ( $1 \times 10^{10} \text{ M}^{-1}$ ) four of these domains have been expressed together in the following way:

15 ~~PL-N and PL-C1 are synthetic oligonucleotides (manufactured by the~~  
 Biomolecular Unit at Lund University (Sweden) in accordance with Applicant's  
 instructions) which have been used to amplify a clonable gene-fragment which is amplified  
 with PCR (Polymerase Chain Reaction) and which codes for four Ig-binding protein L  
 domains (ala-val-glu-asn-B1-B2-B3-B4-lys-lys-val-asp-glu-lys-pro-glu-glu). Amino acids  
 in the protein L-sequence are given for the primer which corresponds to the coded strand  
 (PL-N):

20 PL-N: 5' -GCTCAGCGGCGCCGGTAGAAAATAAGAAGAAACACCAGAAAC-3'  
 / valgluasnlysglugluthrproglu

25 5'-end of this oligonucleotide is homologous with the coded strand in the  
 protein L-gene (emphasized): those codons which code for the last three amino acids in the  
 A-domain (val-glu-asn) are followed by the codons for the first six amino acids in the first  
 of the Ig-binding domains in protein L (B1).

PE-C1: 5'-CAGCAGCA GGATTC TTATTATTCTTCTGGTTTTTCGTCAACTTT  
 CTT-3'

This oligonucleotide is homologous with the opposing non-coding strand in the gene for protein L (the sequence corresponds to the first nine amino acids in domain B5).

DNA-fragments which have been amplified with the aid of PL-N contain the recognition sequence for the restriction enzyme **HpaII** (emphasized) immediately before the codon which is considered to code for the first amino acid (val) in the expressed protein L-fragment. The fragment which is cleaved with **HpaII** can be ligated with DNA (in this case, consisting of the used expression vector pHD389) which has been cleaved with the restriction enzyme **NarI**. The DNA-fragment that has been cleaved with **HpaII** and ligated with vector pHD389, which has been cleaved with **NarI**, will be translated in the correct reading frame. The construction results in translation of an additional amino acid (ala) immediately in front of the first amino acid in protein L.

DNA-fragments which have been amplified with the aid of PL-C1 will contain the recognition sequence for the restriction enzyme **BamHI** (overlined above the sequence) immediately after the sequence which codes for the last amino acid in the expressed protein L-fragment (glu). The vector pHD389 contains a unique recognition sequence for **BamHI** as part of its so-called multiple cloning sequence which follows the **NarI** recognition sequence. DNA-fragments which have been amplified with the aid of PL-C1 will include two so-called stop-codons (emphasized) which results in translation of the fragment inserted in the vector to cease.

The sequence which was considered to be amplified contains no internal recognition sequences for the restriction enzymes **HpaII** or **BamHI**.

## 25 Amplifying and cloning procedures

(PCR) (Polymerase Chain Reaction) was effected with a protocol described by Saiki, R.D. Gelfand, S. Stoffel, S. Scharf, R. Higuchi, G. Horn, K. Mullis and H. Erlich,

1988; Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-49127; PCR was effected in a Hybaid Intelligent Heating-block (Teddington, UK): 100  $\mu$ l of a reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml gelatine, 300  $\mu$ M with respect to each of the deoxynucleotides (dATP, dCTP, dGTP, dTTP), (Pharmacia), 20 pmol of each of the oligonucleotides PL-N and PL-C1, 10  $\mu$ l of a target (template) DNA-solution containing 0.1 mg/ml of chromosomal DNA from *Peptostreptococcus magnus*, species 312. The mixture was covered with mineral oil (Sigma) and DNA was denatured by heating to 98°C for 10 minutes. 2.5 units of AmpliTaq (Perkin Elmer Cetus, Norwalk, CT) were added and PCR was then carried out with 25 cycles consisting of a denaturing step at 94°C for 1 minute, followed by a hybridizing step at 56°C for 1 minute, and finally by an extension step at 72°C for 1 minute. Amplified DNA was analyzed by electrophoresis in agarose gel. The amplified DNA was cleaved with the restriction enzymes **HpaII** (Promega), (8 units/ $\mu$ g amplified DNA) and **BamHI** (Promega), (10 units/ $\mu$ g amplified DNA) at 37°C. The thus amplified and subsequently cleaved DNA-product was isolated by electrophoresis in a 2% (weight by volume) agarose gel (NuSieve agarose, FMC Biproducts) in a TAE-buffer (40 Mm Tris, 20 Mm Na-acetate, 2 Mm EDTA, Ph 8.0). The resulting 930 base-pair fragment was cut from the gel. The DNA concentration in this removed gel-piece was estimated to be 0.05 mg/ml. The agarose-piece containing the cleaved, amplified fragment was melted in a water bath at 65°C, whereafter the fragment was allowed to cool to 37°C. 10  $\mu$ l (0.5  $\mu$ g) of this DNA was transferred to a semimicrotube (Sarstedt) preheated to 37°C, whereafter 1  $\mu$ l of the vector pHD389 was immediately added and cleaved with **NarI** (Promega) and **BamHI**, 1  $\mu$ l 10xligas-buffer (Promega) and 1  $\mu$ l T4 DNA-ligase (Promega; 1 unit/ $\mu$ l). The ligating reaction was then used to transform *E. coli*, strain LE392 that had been made transformation-competent in accordance with the rubidium/calcium-chloride-method as described by Kushner (1978). Molecular biological standard methods have been used in the manipulation of DNA (Sambrook, J.E. Fritsch and T. Maniatis, 1989. Molecular cloning: A laboratory manual. 2nd Ed. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, USA). The cleaving and ligating conditions

recommended by the manufacturer of DNA-ligase and restriction enzymes have been followed in other respects.

### Expression system

The vector pHD389 (see Figure 2) is a modified variant of the plasmid pHD313 (Dalbøge, H.E. Bech Jensen, H. Töttrup, A. Grubb, M. Abrahamson, I. Olafsson and S. Carlsen, 1989. High-level expression of active human cystatin C in *Escherichia coli*. *Gene*, 79: 325-332). The vector, which is replicated in *E. coli* (contains ori = origin of replication from plasmid pUC19) is constructed so that DNA-fragments which have been cloned into the cleaving site of **NarI** will be transcribed and translated downstream of and in the immediate vicinity of the signal peptide (21 amino acids), from envelope-protein **ompA** from *E. coli*. Translation will be initiated from the codon ATG which codes for the first amino acid (methionine) in the signal peptide. This construction permits the translated peptide to be transported to the periplasmic space in *E. coli*. This is advantageous, since it reduces the risk of degradation of the desired product of enzymes occurring intracellularly in *E. coli*. Moreover, it is easier to purify peptides which have been exported to the periplasmic space. Unique recognition sequences (multiple cloning sequences) for several other restriction enzymes, among them **ecoRI**, **SalI** and **BamHI** are found immediately after the **NarI** cleaving site. An optimized so-called Shine-Dalgarno-sequence (also called ribosomal binding site, RBS) is found seven nucleotides upstream from the ATG-codon in the signal sequence from **ompA**, this optimized sequence binding to a complementary sequence in 16S rRNA in the ribosomes and is responsible for the translation being initiated in the correct place. The transcription of such DNA as that which is co-transcribed with the signal sequence for **ompA** is controlled by the  $P_R$ -promotor from coliphage  $\lambda$ . The vector also contained the gene for **cl857** from coliphage  $\lambda$  whose product down-regulates transcription from  $P_R$  (and whose product is expressed constitutively). This **cl857**-mediated down-regulation of transcription from  $P_R$  is heat-sensitive. The transcription regulated from this promotor is terminated with the aid of a so-called rho-independent transcription terminating sequence (forms a structure in DNA which results in

the DNA-dependent RNA-polymerase leaving the DNA-strand) which is placed in the vector immediately downstream of the multiple cloning sequence. The plasmid also carries the  $\beta$ -lactamase gene (from the plasmid pUC19) whose product permits ampicillin-selection of *E. coli* clones that have been transformed by the vector.

## 5 Selection of protein L-producing clones

The transformed bacteria are cultivated, or cultured, on culture plates with an LB-medium which also contained ampicillin in a concentration of 100  $\mu$ g/ml. Cultivation of the bacteria progressed overnight at 30°C, whereafter the bacteria were transferred to an incubator where they were cultivated for a further 4 hours at 42°C. The plates were kept in a refrigerator overnight. On the next day, the colonies were transferred to nitrocellulose filters. Filters and culture plates were marked so as to enable the transferred colonies to be readily identified on respective culture plates. The culture plates were again incubated overnight at 30°C, so that remaining rests of transferred bacteria colonies could again grow. The plates were then kept in a refrigerator. The bacteria in the colonies on the nitrocellulose-impressions were lysed by incubating the filter in 10% SDS for 10 minutes. Filters containing lysed bacteria were then rinsed with a blocking buffer which comprised PBS (pH 7.2) with 0.25% gelatine and 0.25% Tween-20 (four baths, 250 ml each at 37°C), whereafter the filter was incubated with radioactively marked (marked with  $^{125}$ I in accordance with the chloramin-T-method) Ig- $\kappa$ -chains (20 ng/ml in PBS with 0.1% gelatine). The incubation took place at room temperature over a period of 3 hours, whereafter non-bound radioactively marked protein was rinsed-off with PBS (pH 7.2) containing 0.5 M NaCl, 0.25% gelatine and 0.25% Tween-20 (four baths, 250 ml each at room temperature). All filters were exposed to X-ray film. Positive colonies were identified on the original culture plate. Clones which reacted with Ig- $\kappa$ -chains were selected and analyzed with respect to the size on the DNA-fragment introduced in the vector. One of these clones was selected for the production of protein L, pHDL. The DNA introduced from this clone into plasmid pHD389 was sequenced. The DNA-sequence was found to be in full agreement with corresponding sequences (B1-B4 and 21 bases in B5) in



the gene for protein L from *Peptostreptococcus magnus*, strain 312. The size and binding properties of the protein produced by clone pHDL was analyzed with the aid of SDS-PAGE (see Figure 8), dot-blot experiment (see Figure 9) and competitive binding experiments.

## 5 Production of protein L

Several colonies from a culture plate with *E. coli* pHDL were used to inoculate a preculture (LB-medium with an addition of 100 mg/l ampicillin), which was cultured at 28°C overnight. On the following morning, the preculture was transferred to a larger volume (100 times the volume of the preculture) of fresh LB-medium containing  
 10 ampicillin (100 mg/l) and was cultured in shake-flasks (200 rpm), (or fermentors) at 28°C. The culture temperature was raised to 40°C (induction of transcription) when the absorbency value at 620 nm reached 0.5. Cultivation then continued for 4 hours (applied solely to cultivation in shake-flasks). Upon completion of the cultivation process, the bacteria were centrifuged down. The bacteria were then lysed with an osmotic shock  
 15 method at 4°C (Dalbøge et al., 1989 supra). The lysate was adjusted to a pH = 7. Remaining bacteria rests were then centrifuged down, whereafter the supernatant was purified on IgG-sepharose in accordance with earlier described protocol for protein G and protein L (U. Sjöbring, L. Björck and W. Kastern. 1991. Streptococcal protein G: Gene structure and protein binding properties. J. Biol. Chem. 266: 399-405; W. Kastern, U.  
 20 Sjöbring and L. Björck. 1992. Structure of peptostreptococcal protein L and identification of a repeated immunoglobulin light chain-binding domain. J. Biol. Chem. 267 (18):12820-5.

The expression system gave about 20 mg/l of protein L when cultivation in shake-flasks. The culture was deposited at DSSM, Identification Reference DSSM *E. coli*  
 25 LE392/pHDL.

Example 2Cloning and expression of protein LG**Construction of oligonucleotides (primers) for amplifying sequences which code for protein LG****5 Protein L**

It has been found that a protein L-peptide (expressed in *E. coli*) constructed of the sequence ala-val-glu-asn domain B1 (from protein L) will bind to the light chains of the immunoglobulins (Kastern, Sjöbring and Björck, 1992, J. Biol. Chem. 267 (18):12820-5). Since the affinity of this simple domain to Ig is relatively low ( $1 \times 10^{-7} \text{ M}^{-1}$ ) and since  
 10 the naturally occurring protein L, which is comprised of several mutually similar domains (B1-B5) has a higher affinity to Ig ( $1 \times 10^{10} \text{ M}^{-1}$ ), four of these domains have been expressed together in the following way:

*unsp* ~~PL-N~~ and ~~PL-C2~~ are synthetic oligonucleotides (manufactured at the Biomolecular Unit at Lund University (Sweden) in accordance with Applicant's  
 15 instructions) which were used, with the aid of PCR (Polymerase Chain Reaction) to amplify a clonable gene fragment, called B1-4, which codes for four Ig-binding protein L domains (ala-val-glu-asn-B1-B2-B3-B4-lys-lys-val-asp-glu-lys-pro-glu-glu):

PL-N: 5' -GCTCAGGCGGCGCCGGTAGAAAATAAAGAAGAAACACCAGAAAC-3'

valgluasnlysglugluthrproglu

20 ~~P1-C2: 5' -CAGCAGCAGCCATGGGTTCTTCTGGTTTTTCGTCAACTTTCTTA-3',~~

Amino acids have been shown under corresponding triplets in the coded strand. DNA-fragments which have been amplified with the aid of PL-N contain the recognition sequence for the restriction enzyme **HpaII** immediately upstream of the triplet which codes for the first amino acid (val) in the expressed protein L-fragment. The  
 25 fragment that has been cleaved with **HpaII** can be ligated with DNA (in this case, the used

expression vector pHD389) which has been cleaved with **NarI**. The construction results in translation of an extra amino acid (ala) immediately upstream of the first amino acid in the protein L-fragment. The DNA-fragment that has been amplified with the aid of PL-C2 will contain the recognition sequence for the restriction enzyme **NcoI** (emphasized) immediately downstream of the sequence which codes for the last amino acid in the expressed protein L-fragment (glu). Amplified fragments which have been cleaved with **NcoI** can be ligated to the **NcoI**-cleaved, PCR-generated protein-asp-CDC-met-fragment (see below).

### Protein G

<sup>10</sup> ~~It is known that a simple C-domain from protein G will bind to IgG (B. Guss, M. Eliasson, A. Olsson, M. Uhlen, A.-K. Frej, H. Jörnvall, I. Flock and M. Lindberg. 1986. Structure of the IgG-binding regions of streptococcal protein G. EMBO. J. 5: 1567-1575). The strength at which a simple C-domain binds to IgG is relatively low ( $5 \times 10^7$  M<sup>-1</sup>). A fragment which consists of two C-domains with an intermediate D-region having a length of 15 amino acids, however, has a considerably higher affinity to IgG ( $1 \times 10^9$  M<sup>-1</sup>). CDC-N and CDC-C are oligonucleotides which have been used as PCR-primers to amplify a clonable DNA-fragment, designated CDC, which codes for two IgG-binding protein G-domains (pro-met-asp-CDC-met).~~

<sup>20</sup> CDC-N: GG CCATGG ACACTTACAAATTAATCCTTAATGGT  
metaspthrtyrlysleuileleuasngly

~~CDC-C: C AGGTGG ACTTATTACATTTTCAGTTACCGTAAAGGTCTTAGT~~

Amino acids in the resultant sequence have been shown beneath the primer of the coding strand. DNA-fragments which have been amplified with the aid of CDC-N contain the recognition sequence for the restriction enzyme **NcoI** (marked with a line above the sequence). Cleaved amplified fragments can be ligated with the fragment that has been amplified with the aid of PL-C2 and then cleaved with **NcoI**. The fragment will therewith

be translated to the correct reading frame. DNA-fragments which have been amplified with the aid of CDC-C will contain two so-called stop codons (emphasized) which terminate translation. The recognition sequence for the restriction enzyme **Sall** (marked with a line above the sequence) follows immediately afterwards, this sequence also being  
 5 found in the expression vector pHD389 (see Figure 1).

Those sequences which code for the binding properties of protein L (B1-B5) and for protein G (CDC) respectively contain no internal recognition sequences for the restriction enzymes **HpaII**, **Sall** or **NcoI**.

### Amplification and cloning procedures

10 PCR (Polymerase Chain Reaction) was carried out in accordance with a protocol described by Saiki et al., 1988; PCR was carried out in a Hybaid Intelligent Heating-block (Teddington, UK): 100 µl of the reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 100 µg/ml gelatine, 300 µM with respect to each of the deoxynucleotides (dATP, dCTP, dGTP, dTTP), (Pharmacia). In order to amplify  
 15 sequences which code for the light-chain binding parts of protein L, there were added 20 pmol of each of the oligonucleotides PL-N and PL-C2, and 10 µl of a DNA-solution which contained 0.1 mg/ml of chromosomal DNA from *Peptostreptococcus magnus*, strain 312. By way of an alternative, 20 pmol were added to each of the oligonucleotide pairs CDC-N and CDC-C and 10 µl of a DNA-solution which contained 0.1 mg/ml of chromosomal  
 20 DNA from a group C streptococcus strain (*Streptococcus epuisimilis*) called C40 (U. Sjöbring, L. Björck and W. Kastern. 1991. Streptococcal protein G: Gene structure and protein binding properties. J. Biol. Chem. 266: 399-405 or with **NcoI** and **Sall** (10 U/µg PCR-product), (for CDC) at 37°C. The thus amplified and subsequently cleaved DNA-fragments were then separated by electrophoresis in a 2% (weight by volume) agarose gel  
 25 (NuSieve agarose, FMC Bioproducts) in a TAE-buffer (40 mM Tris, 20 mM Na-acetate, 2 mM EDTA, pH 8.0). The resultant fragments, 930 bp (for B1-4) and 390 bp (for CDC) were cut from the gel. The concentration of DNA in the thus separated gel pieces was estimated to be 0.05 mg/ml. The agarose pieces cut from the gel and containing the

cleaved, amplified fragments (B1-4 and CDC) were melted in a water bath at 65°C, whereafter they were allowed to cool to 37°C. 10 µl (0.5 µg) of this DNA were transferred to a semi-microtube (Sarstedt), preheated to 37°C, whereafter 1 µl of the vector pHD389 which had been cleaved with **NarI** and **Sall** were added. 1 µl 10 x ligase buffer (Promega) and 1 µl T4 DNA-ligase (1 unit/µl) were also added. The ligating reaction was permitted to take place at 37°C for 6 hours. The cleaving and ligating conditions recommended by the producer of DNA-ligase and restriction enzymes (Promega) were followed in other respects. The ligating reaction was then used to transform *E. coli*, strain LE392, which had been made competent in accordance with the rubidium-chloride/calcium-dichloride method as described by Kushner (1978). Manipulation of DNA was effected in accordance with molecular biological standard methods (Sambrook et al., 1989).

### Expression system

The vector pHD389 (see Figure 2) is a modified variant of the plasmid pHD313 (Dalbøge et al., 1989). The vector which was replicated in *E. coli* (contains origin of replication from plasmid pUC19) is constructed such that DNA-fragments which have been cloned in the cleaving site for **NarI** will be expressed immediately after, or downstream, of the signal peptide (21 amino acids) from the envelope protein **ompA** from *E. coli*. Translation will be initiated from the ATG-codon which codes for the first amino acid (methionine) in the signal peptide. The construction with an *E. coli*-individual signal sequence which precedes the desired peptide enables the translated peptide to be transported to the periplasmic space in *E. coli*. This is beneficial since it reduces the risk of degradation of the desired product through the intracellular occurrent enzymes of *E. coli*. Furthermore, it is easier to purify peptides which have been exported to the periplasmic space. Unique recognition sequences (multiple cloning sequences) for several other restriction enzymes, among them **EcoRI**, **Sall** and **BamHI** are present immediately downstream of the **NarI** cleaving site. An optimized so-called Shine-Dalgarno sequence (also called ribosomal binding site, RBS) is found seven nucleotides upstream of the ATG-codon in the signal sequence from **ompA**, this optimized Shine-Dalgarno sequence

binding to a complementary sequence in 16S rRNA in the ribosomes and in a manner to decide that the translation is initiated in the correct place. The transcription of such DNA as that which is co-transcribed with the signal sequence for **ompA** is controlled by the  $P_R$ -promotor from coliphage  $\lambda$ . The vector also contains the gene for cI857 from coliphage  $\lambda$ ,  
 5 the product of which regulates-down transcription from  $P_R$  and the product of which is expressed constitutively. This cI857-mediated down-regulation of transcription from  $P_R$  is heat-sensitive. Transcription which is regulated, or controlled, from this promotor will be terminated with the aid of a so-called rho-independent transcription terminating sequence which is inserted in the vector immediately downstream of the multiple cloning site. The  
 10 plasmid also carries the gene for  $\beta$ -lactamase (from the plasmid pUC19), the product of which permits ampicillin-selection of *E. coli* clones that have been transformed with the vector.

### **Selection of protein LG-produced clones**

The transformed bacteria are cultivated on culture plates with LB-medium  
 15 which also contained ampicillin in a concentration of 100  $\mu$ g/ml. The bacteria were cultivated overnight at 30°C, whereafter they were transferred to a cultivation cabinet (42°C) and cultured for a further four (4) hours. The plates were stored in a refrigerator overnight. On the following day, the colonies were transferred to nitrocellulose filters. The filters and culture plates were marked, so that the transferred colonies could later be  
 20 identified on the culture plate. The culture plates were again incubated overnight at 30°C, so that rests of transferred bacteria colonies remaining on the plates could again grow. The plates were then stored in a refrigerator. The filter was incubated in 10% SDS for 10 minutes, so as to lyse the bacteria in the colonies on the nitrocellulose impression. Filters containing lysed bacteria were then rinsed with a blocking buffer consisting of PBS (pH  
 25 7.2) with 0.25% gelatine and 0.25% Tween-20 (four baths of 250 ml at 37°C), whereafter the filter was incubated with radioactively (marked with  $^{125}$ I according to the chloramine-T-method) marked Ig- $\kappa$ -chains (20 ng/ml) in PBS with 0.1% gelatine). The incubation process took place at room temperature for four (4) hours, whereafter non-bound

radioactively marked protein was rinsed-off with PBS (pH 7.2) containing 0.5 M NaCl, 0.25% gelatin and 0.25% Tween-20 (four baths, 250 ml each at room temperature). All filters were exposed to X-ray film. Positive colonies on the original culture plate were identified. A number of positive colonies were recultivated on new plates and new colony-

5 blot experiments were carried out with these plates as a starting material with the intention of identifying *E. coli* colonies which bind IgG Fc. These tests were carried out in precisely the same manner as that described above with respect to the identification of *E. coli*-colonies which expressed Ig light-chain-binding protein, with the exception that a radioactively marked ( $^{125}\text{I}$ ) IgG Fc (20 ng/ml) was used as a probe. Clones which reacted

10 with both proteins were selected and analyzed with regard to the size of the DNA-fragment introduced in the vector. One of these clones was chosen for production of protein LG, pHDLG. The DNA taken from this clone and introduced into plasmid pHD389 was sequenced. The DNA-sequence exhibited full agreement with corresponding sequences (B1-B4 and 21 bases in B5) in the gene for protein L from *Peptostreptococcus magnus*,

15 strain 312, and with CIDC2 sequence in group C streptococcus strain C40. The size and binding properties of the protein produced from clone pHDLG was analyzed with the aid of SDS-PAGE (see Figure 8), dot-blot experiment (see Figure 10) and competitive binding experiments.

### Production of protein LG

20 Several colonies from a culture plate with *E. coli* pHDLG were used to inoculate a preculture (LB-medium with an addition of 100 mg/l ampicillin) were cultivated at 28°C overnight. In the morning, the preculture was transferred to a larger volume (100 times the volume of the preculture) of fresh LB-medium containing ampicillin (100 mg/l) and was cultivated in vibrating flasks (200 rpm), (or fermenters) at 28°C. When

25 an absorbance value of 0.5 was reached at 620 nm, the cultivation temperature was raised to 40°C (induction of transcription). The cultivation process was then continued for 4 hours (applies only to cultivation in vibrated flasks). The bacteria were centrifuged down upon termination of the cultivation process. The bacteria were then lysed at 4°C in

accordance with an osmotic shock method (Dalbøge et al., 1989). The lysate was adjusted to a pH of 7. Remaining bacteria rests were centrifuged down and the supernatant then purified on IgG-sepharose, in accordance with the protocol earlier described with reference to protein G and protein L. (Sjöbring et al., 1991, Kastern et al., 1992).

- 5                   The expression system gave about 30 mg/l of protein LG when cultivation in vibrated flasks. A deposition has been made at DSSM, Identification Reference DSSM *E. coli* LE392/pHDLG.

### Example 3

#### **Analysis of the binding properties of protein LG**

##### **10 Western Blot**

- Protein G (the ClDC2-fragment), protein L (four B-domains) and protein LG were isolated with SDS-PAGE (10% acrylamide concentration). The isolated proteins were transferred to nitrocellulose membranes in three similar copies (triplicate). Each of these membranes was incubated with radioactively marked proteins (20 ng/ml: one of the
- 15   membrane-copies was incubated with human polyclonal IgG, another with human IgG Fc-fragment and the third with isolated human IgG  $\chi$ chains. Non-bound radioactively marked proteins were rinsed off and all filters were then exposed to X-ray film.

##### **Slot-blot**

- Human polyclonal Ig-preparations and Ig-fragments were applied with the
- 20   aid of a slot-blot apparatus on nitrocellulose filters in given quantities (see Figure 10) on three similar copies. Each of these membranes was incubated with radioactively marked proteins (20 ng/ml). One of the membrane copies was incubated with protein LG, another with protein L and the third with protein G. Non-bound radioactively marked proteins were rinsed-off and all filters were then exposed to X-ray film,

- 25                   The results are shown in Figures 9 and 10.

Other binding experiments have been carried out, with the following results:



TABLE

Binding of the proteins G, L and LG to immunoglobulins.

Binding protein:	G	K <sub>a</sub>	L	K <sub>a</sub>	LG	K <sub>a</sub>
Immunoglobulin						
<u>Human:</u>						
Polyclonal IgG <sup>*</sup>	+	67 (10)	+	9.0	+	20
IgG subclasses						
IgG <sub>1</sub>	+	2.0	+		+	
IgG <sub>2</sub>	+	3.1	+		+	
IgG <sub>3</sub>	+	6.1	+		+	
IgG <sub>4</sub>	+	4.7	+		+	
IgG fragment						
Fc <sup>*</sup>	+	6.0 (0.5)	-		+	
F(ab') <sub>2</sub> <sup>*</sup>	+	0.4 (0.2)	+		+	
kappa	-		+	1.5	+	
lambda	-		(-) <sup>#</sup>			
Other Ig-classes						
IgM	-		+	11.6	+	
IgA	-		+	10.4	+	
IgE	-		+		+	
IgD	-					
<u>Other Species:</u>						
Polyclonal						
Monkey	+		+		+	
Rabbit						
IgG	+	70	+	0.074	+	
IgG-Fc	+	3.0	-		+	
IgG-F(ab') <sub>2</sub> +	0.44			+		
Mouse	+	41	+	2.6	+	
Rat	+	1.5	+	0.39	+	
Goat	+	14	-		+	

Binding protein:		G	K <sub>a</sub>	L	K <sub>a</sub>	LG	K <sub>a</sub>
Immunoglobulin							
Bovine	IgG <sub>1</sub>	+	3	-		+	
	IgG <sub>2</sub>	+	2	-		+	
Horse		+		-		+	
Guinea Pig		+		+		+	
Sheep		+		-		+	
Dog		+		-		+	
Pig		+		+		+	
Hamster		+					
Cat		-		-			
Hen		-		-			
Monoclonsals <sup>&amp;</sup>							
Mouse							
	IgG <sub>1</sub>	+		+		+	
	IgG <sub>2a</sub>	+		+		+	
	IgG <sub>2b</sub>	+				+	
	IgG <sub>3</sub>	+				+	
	IgM	-		+		+	
	IgA	-		+		+	
Rat							
	IgG <sub>2a</sub>	+		+		+	
	IgG <sub>2b</sub>	+				+	
	IgG <sub>2c</sub>	+				+	

K<sub>a</sub> = affinity constant (M<sup>-1</sup>). \*The numerals within parenthesis disclose the affinity of a recombinant protein G comprised of two IgG-binding domains. #A weak bond to lambda chains exists. Binding to P1 and PLG depends on the type of light chain of Ig.

It will thus be seen that the synthesized hybrid protein LG has a broad  
5 binding activity/specificity.